Immune Complexes in Cystic Fibrosis

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Summary

Circulating immune complexes were detected in serum and sputum of patients with cystic fibrosis (C.F.). There were extensive deposits of immunoglobulins and complement immune complexes in several of the C.F. organs, especially the respiratory and gastrointestinal tracts, but not in the kidneys. Significant concentrations of IgG and of complement complexes could be eluted from the lungs of the C.F. patients but not from those of controls. Studies involving immunoabsorption, autoradiography, and molecular sieving through Sephadex G-200 columns identified both bovine serum albumin and staphylococcal α-haemolysin as two of the antigens present in the immune complexes. The sedimentation constant of the immune complexes was about 8S to 11S. The clinical significance of these immune complexes and the wide variety of antibodies detected in C.F. patients are discussed.

Introduction

Circulating serum autoantibodies to human pancreas in children with cystic fibrosis (C.F.) have been reported by Murray and Thal (1960), and local autoantibodies to lungs from C.F. patients at necropsy have been shown in their sputum by Stein *et al.* (1964). In addition, a variety of serum precipitations have been detected in a high percentage of C.F. patients (Burns and May, 1967; McCarthy *et al.*, 1969). In our previous study not only were a wide variety of precipitating antibodies detected in the serum of C.F. patients but also they were found in much higher concentrations and numbers in the corresponding sputum (Wallwork *et al.*, 1974). These observations prompted us to investigate the occurrence of immune complexes in C.F. patients.

Patients and Methods

We investigated sputum, blood, and biopsy and necropsy specimens from well-documented patients with cystic fibrosis. Necropsy specimens were obtained from two girls aged 8½ years and 11 years, two boys aged 6 months and 3 years, 10 adults with bronchopneumonia, and one child who died as a result of a road accident. Biopsy material was obtained from a 6-month-old child with C.F.

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Antigens.—The following antigens, obtained either from Bencard, Brentford, or prepared locally, were used for investigating the occurrence of antibodies: 1, bovine serum albumin (B.S.A.); 2, pooled human seminal fluid; 3, staphylococcal α-haemolysin; 4, *Aspergillus fumigatus;* 5, *Pseudomonas aeruginosa;* 6, *Brucella abortus;* 7, egg white; 8, *Dermatophagoides pteryssinus;* 9, human colostrum; 10, human urine from a patient with multiple myelomatosis; 11, cows' milk (pasteurized); 12, wheat grain; 13, mixed moulds; 14, mixed bacteria; 15, *Haemophilus influenza;* 16, *Klebsiella pneumoniae;* 17, *Streptococcus pneumoniae* type 1; 18, *Dermatophagoides farinae;* and human serum.

Immunofluorescence.—The immunofluorescence technique for showing antigen antibody complexes was modified from the method of Allison *et al.* (1969). Cryostat sections 6 μm thick were fixed in air, and after washing for two hours in phosphate buffered saline pH 7·2 to remove any immunoglobulins other than those bound in immune complexes they were stained for 30 minutes with either fluorescein isothiocyanate (FITC) protein conjugate alone or in double staining with rhodamine RB 200 protein conjugate.

The following FITC conjugates were used: monospecific antihuman IgG, IgA, IgM, IgE, C1q, C3, and C4. The RB 200-labelled antihuman sera were IgG, IgE, and IgM.

Blocking experiments and specificity staining were carried out with FITC-labelled sheep anti-rabbit immunoglobulins and sheep antihuman immunoglobulins.

The fluorescence-labelled antisera and other antisera were obtained from the Wellcome Laboratories, Beckenham, Kent; Behringwerke Ag, Marburg, Lahn; Meloy Laboratories, Virginia, U.S.A.; and Cappel Laboratories, Dowingtown, Pennsylvania, U.S.A. The fluorescence-stained sections were examined with a Leitz Orthoplan microscope equipped with incident fluorescent light provided by an HBO-200 W/4 super pressure mercury lamp source. The intensity of the fluorescence staining was graded as follows: negative (−), those cryostat sections without any visible fluroescence; minimal (+) those sections with pale and sparse areas of fluorescence; moderate (++), sections with brighter and more regular staining; intense (+++), those in which most areas of the sections were brightly fluorescent; and very intense (++++), when the entire section under the objective field was extensively fluorescing. In all specimens at least three sections were stained and examined with each antiserum and each stained slide was assessed for fluorescence by two independent observers.

Immunoglobulin quantitation was by the method of Mancini *et al.* (1965). IgE quantitation was by the radioimmunoassay method of Johansson *et al.* (1968).

Immunoelectrophoresis was performed by the method of Grabar and Burtin (1964), and the precipitins in sputum were identified by counter immunoelectrophoresis.

Autoradiography.—Sputum sol phase from C.F. patients and from controls was allowed to react from the centre wells in 1·5% agar gel in phosphate buffered saline pH 7·2 against antihuman monospecific and polyvalent IgG, IgA, or IgM and against polyvalent anti-whole human serum placed in the peripheral wells. The control wells in the agar plate contained saline, staphylococcal α-haemolysin, and normal sheep serum since the various anti-human sera used in this experiment were raised in sheep. After immunodiffusion had proceeded for 48 hours the immunoplates containing the precipitin lines were washed with constant stirring for 48 hours in six changes of 3·0% saline. Then 100 μl of ^{125}I-B.S.A. (8·2 μg B.S.A.) was placed

in the centre well and allowed to diffuse to investigate whether this radiolabelled antigen would react with any of the complexes formed between the antibody B.S.A. in the sputum and the antiglobulin—that is, one of the antihuman immunoglobulins. The immunoplates were again washed extensively to remove excess unbound labelled ^{125}I-B.S.A., dried, and investigated for positive immune complexing by means of autoradiography.

Identification of Antibody Class of Complexes.—To 1·0 ml of sputum or to 0·5 ml of serum in a test tube from patients with C.F. was added 100 µl of antihuman IgG, IgA, IgM, anti-whole serum, or saline at weekly intervals. These were allowed to stand at 4°C for one week, after which the tubes were centrifuged to remove any precipitate. The supernatants were examined weekly for eight weeks by counter immunoelectrophoresis for remaining antibodies to B.S.A. or to staphylococcal α-haemolysin.

Sequential Estimation of Precipitin.—Four patients provided a total of 28 sputum specimens and nine serum specimens over a period of one year. Fifty other children with C.F. provided at least two specimens each of sputum and serum. Each of these specimens was tested by counter immunoelectrophoresis for the presence of precipitating antibodies to the nineteen different antigens.

Isolation of Immune Complexes.—Copious deposits of immune complexes were precipitated from sputum of C.F. patients by either deionized H_2O or by low ionic strength buffers. After resuspension and molecular sieving through Sephadex G-200 columns these immune complexes emerged from the columns with the void volume of the eluting fluid and were then tested for their immunoglobulin and complement contents.

Elution of Immune Complexes from Lungs and Pancreas.—About 1·0 g each of the lungs and pancreas taken at necropsy from two C.F. patients and one control patient was homogenized in cold phosphate buffered saline pH 7·2 and the homogenate washed seven times with the same buffer according to the method of Allison *et al.* (1969). The sediment was eluted with 1·0 ml of 0·1-M Sorensen citric acid-NaOH-HCl buffer pH 2·5 then neutralized and allowed to stand for 18 hours. In the first 2 hours aliquots were removed every 5 to 10 minutes for testing in double immunodiffusion in agar gel against antihuman serum to the following proteins: IgG, IgA, IgM, C3, C4, and C5.

Complement Components.—Fresh samples of plasma taken on the same day from three C.F. patients and one control subject were tested in two-dimensional immunoelectrophoresis for the altered form of C3 complement. Total concentration of C3 and C4 was also performed on serum from 40 patients with C.F. and 59 controls.

Physiochemical Properties of S IgA.—Sputum for S IgA was purified by means of DEAE cellulose chromatography and by gel filtration through columns of Sephadex G-200. This was followed by hydrolysis of the S IgA by means of mercaptoethanol and sodium dodecyl sulphate and subsequent electrophoresis through polyacrylamide disc gels. The various components were compared with corresponding fragments of normal human colostral S IgA.

Results

The immunofluorescence staining of the biopsy specimen from the gut of a 6-month-old child (case 1) with C.F. before any treatment had begun showed extensive IgG, IgA, and IgM immune complexes with moderate amounts of IgE and C1q complexes in the submucosal areas of the gut wall. Some areas appeared with focal staining but most had linear membranous immunofluorescence. The typical extensive nature of the immune complexes in various organs of the body at necropsy in one patient (case 2) with C.F. is shown in table I. The immune complexes consisted mainly of IgG, IgM, C1q, C3, and C4 with smaller amounts of IgA and IgE. The lungs, the trachea, and the pancreas contained by far the most extensive deposits of immune complexes followed by the gut and thymolymphatic organs and then the liver. No immune complexes of any significance were detected in any of the kidneys examined from C.F. patients. The extensive linear and membranous deposits of immune complexes in some of the organs examined are shown in figs. 1-4. The trachea contained extensive linear immunofluorescence staining in the ganglion cell wall and

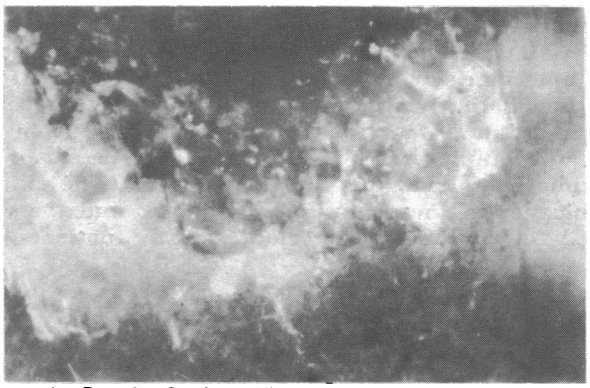

FIG. 1—Case 2a. Section of lung taken at necropsy stained with FITC-conjugqte against human IgG showing continuous linear fluorescence along walls of air spaces of middle lobe of lung.

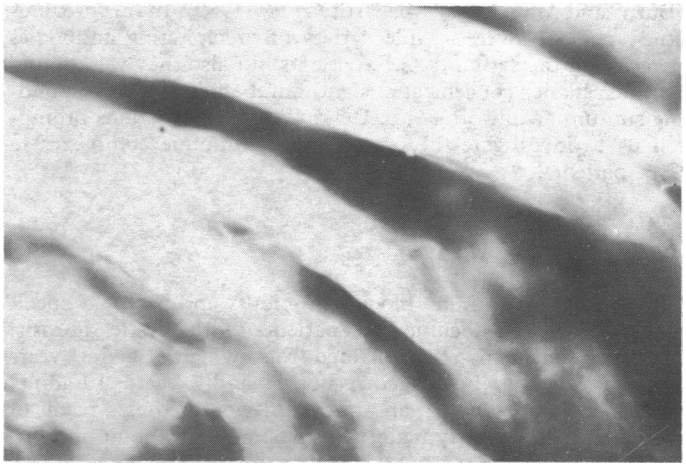

FIG. 2—Case 2. Section of trachea taken at necropsy stained with FITC-conjugate against human IgG showing extensive fluorescence of most areas.

TABLE I—*Results of Immunofluorescence Staining with Labelled FITC Monospecific Antihuman Immunoglobulins and Complement Components of Different Necropsy Sections of one C.F. Patient (Case 2)*

	IgG	IgA	IgM	IgE	C1q	C3	C4
Kidney	−	−	−	−	−	+	−
Spleen	+ + + +	+ + +	+ +	+ +	+ +	+ +	+ +
Thymus	+ + + +	+ +	+ + +	N.D.	+ + + +	+ + +	+ +
Liver	+ + + +	+ +	−	+	+	+ + +	N.D.
Stomach	+ + + +	+	+ +	N.D.	+	+ + +	+ + +
Duodenum	+ + + +	N.D.	+ + +	N.D.	+ + +	N.D.	N.D.
Pancreas	+ + + +	+ + +	+	+ + +	+	+ +	+ + +
Lung	+ + + +	+ + +	+ +	+ + +	+ +	+ +	+ + + +
Trachea	+ + + +	+ + +	+ + + +	+ + + +	+ +	+ + + +	+ + +

N.D. = Not done.

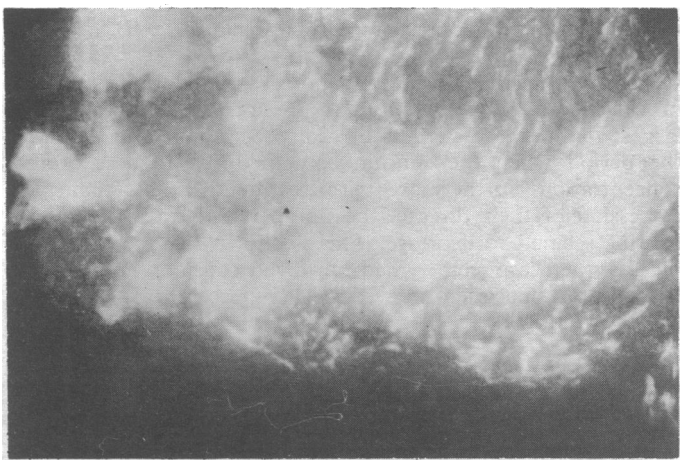

FIG. 3—Case 1. Section of gut biopsy specimen stained with FITC-conjugate against human IgE showing coarse granular fluorescence staining.

FIG. 4—Case 2. Section of stomach taken at necropsy stained with FITC-conjugate of human IgG showing linear membranous fluorescence.

myelin nerve fibres not only by IgG and IgM but also by the IgE as well as by C3 and C4. One other patient (case 3) had rather extensive infiltration of the lungs with IgA-staining plasma cells but few IgG-staining plasma cells. Positive immunofluorescence staining was also observed in the cryostat sections from necropsy lung specimens of 10 adult patients who had a history of either bronchitis or bronchopneumonia. Nevertheless, no immunofluorescence staining was detected in lung sections from a 10-year-old child who had died from a road accident.

Double Immunofluorescence.—Cryostat sections of lungs and pancreas stained first with rhodamine RB 200 conjugate then with FITC conjugate showed immune complex deposits which were stained simultaneously with two different classes of immunoglobulin antisera (fig. 5 and table II). Furthermore, the resultant immunofluorescence patterns were significantly brighter with the double-staining than with the single-staining method. Control specimens from one non-C.F. patient did not show such enhanced immunofluorescence by double staining.

Specificity of Immunofluorescence.—Sheep anti-rabbit immunoglobulin-FITC conjugate did not stain any of the cryostat sections of the C.F. organs nor of the control organs, indicating the specificity of the fluorescence detected in the C.F. organs by the antihuman protein conjugates.

Immunoglobulins.—A transient decrease of the plasma IgA and IgG was observed in 20% of the C.F. patients, and limited follow-up studies showed that such patients subsequently acquired a higher than normal serum IgA and IgG concentration, especially those who developed infections. Total serum IgM concentration was normal in most patients investigated except for two patients who had serum IgM of 0·26 g/l and 0·36 g/l. The Sephadex G-200 gel filtration pattern of a sputum specimen from a patient with C.F. is shown in fig. 6. The lack

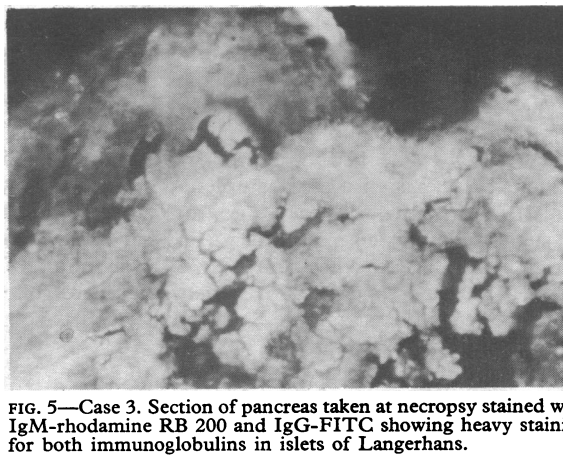

FIG. 5—Case 3. Section of pancreas taken at necropsy stained with IgM-rhodamine RB 200 and IgG-FITC showing heavy staining for both immunoglobulins in islets of Langerhans.

TABLE II—*Results of Double Immunofluorescence Staining, First with RB 200-labelled Protein followed by FITC-labelled Protein of Necropsy Sections of Patient in Case 2*

	Test 1		Test 2	
	IgM—RB 200	IgG—FITC	IgE—RB 200	C1q—FITC
Pancreas ..	+ +	+ + + +	+ +	+ +
Lung	+ +	+ + + +	+ +	+ + + +

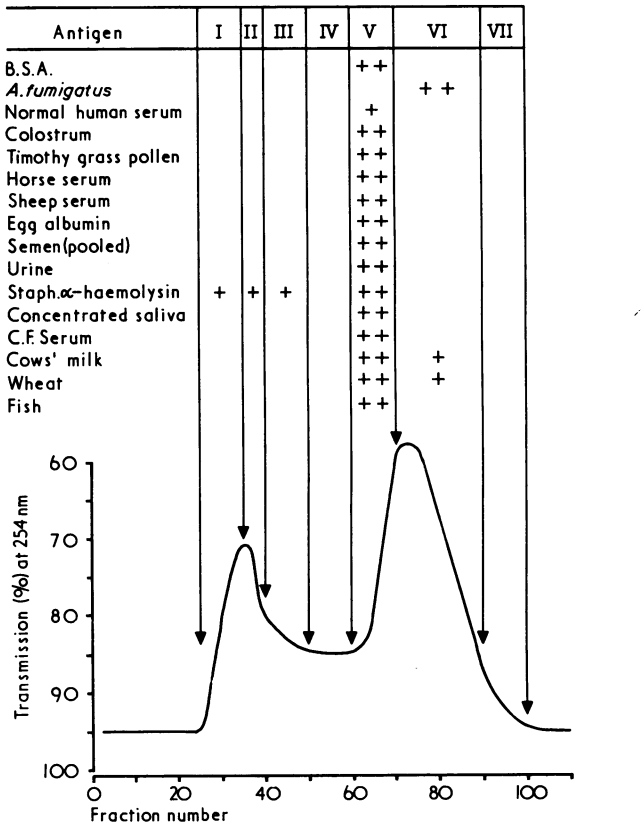

FIG. 6—Results of Sephadex G-200 gel filtration of sputum from patient with cystic fibrosis and of the counter immunoelectrophoresis of eluates against different antigens. Most of the positive precipitin is in IgG region and one only is positive in S IgA region.

of a wide variety of antibodies of the IgA class in the sputum as well as the inability of the sputum antibodies to bind to affinity-labelled columns in preliminary experiments may be an index of low-affinity binding antibodies in C.F. Failure to agglutinate both sensitized and unsensitized sheep red blood cells by C.F. sputum containing high titres of precipitating antibodies seems to be further evidence of their low-affinity binding and their existence as incomplete antibodies. Physicochemical studies of the sputum S IgA molecules from a C.F. patient suggested a faster migrating S IgA molecule in C.F. sputum than in human colostrum (fig. 7) though hydrolysis with mercaptoethanol and sodium dodecyl sulphate followed by disc gel electrophoresis showed that all the components of the S IgA system were present.

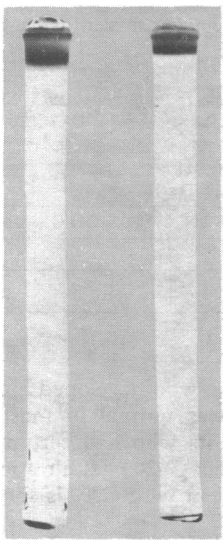

FIG. 7—Results of polyacrylamide disc gel electrophoresis of purified S IgA from sputum of C.F. patient with cystic fibrosis (left) in comparison to the S IgA from normal human colostrum (right).

Autoradiography showed that the ^{125}I-B.S.A. antigen complexed with the precipitin lines formed between either anti-IgM or anti-IgG—anti-B.S.A. in C.F. sputum (fig. 8).

Antibody Class of the Complexes.—Immunoabsorption of serum and sputum of C.F. patients showed that only the IgG class of antihuman serum was able to remove one of the three *Staphylococcus aureus* immunoprecipitin lines from the C.F. serum whereas absorption with Staphylococcal α-haemolysin antigen removed two of these staphylococcal precipitin lines.

Monospecific as well as polyvalent antiserum to human IgG, IgA, and IgM and bovine serum albumin were capable of completely absorbing out the immunoprecipitins to B.S.A. from C.F. sputum.

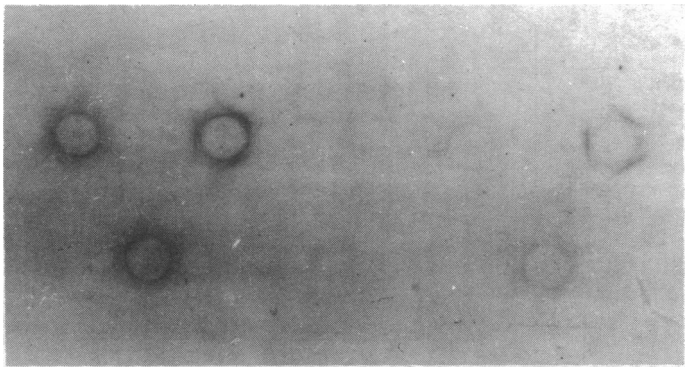

FIG. 8—Case 4. Results of autoradiograph of precipitins in sputum showing positive complexing of ^{125}I-B.S.A. antigen with antihuman IgM-B.S.A. antibody (left) and with antihuman IgG-B.S.A. antibody (right). No precipitin complexes were observed with anti-IgA nor with sheep serum in control wells.

Sequential Estimation of Precipitins.—Sequential estimation of a wide variety of precipitins in the sputum showed that the largest number and highest titre of precipitins coincided with the period when the sputum contained the highest protein concentration. Furthermore, with treatment of the patients and regression of their disease, the number and titre of the precipitins seemed to decrease. This is shown for one patient in fig. 9. When last seen he had improved considerably, and the last sputum specimen showed positive precipitin against the only two antigens.

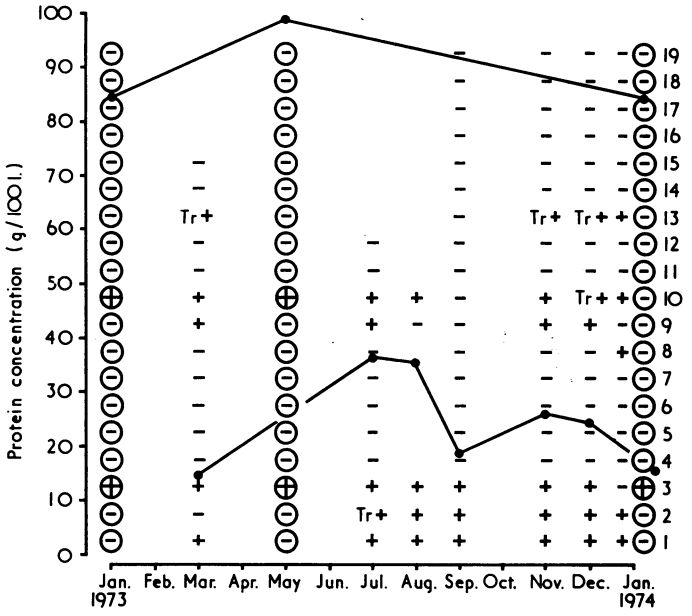

FIG. 9—Case 5. Sequential estimation 1973-4 of precipitins in three serum (open circles) and seven sputum specimens from patient with cystic fibrosis. Numbers 1-19 represent antigens (see Patients and Methods). Positive (+) and negative (−) signs within circles indicate that serum specimen gave positive or negative precipitin reaction against respective antigen. Solid lines represent total protein concentrations in serum (upper graph) and sputum (lower graph). Note tendency towards decrease in total-protein concentration and numbers of positive precipitin reactions in both serum and sputum with treatment of patient.
Tr = Trace.

Elution of Immune Complexes.—The euglobulin fraction isolated with low ionic strength solutions from the sputum of patients with C.F. contained positive precipitin reactions against anti-IgG, IgM, IgA, C3, and C4. In addition, double immunodiffusion in agar gel of the eluted samples from homogenized necropsy specimens of the lungs and pancreas of two patients (cases 2 and 3) and one control against various antisera to human proteins showed that the lungs of both patients contained about 100-200 mg of IgG complex per g of wet weight of lung. About 30 mg of C3 was eluted from the lung of one C.F. patient (case 3). No immune complexes could be eluted from C.F. pancreas nor from one of the control lungs tested.

Complement Components.—Fresh plasma from three patients with C.F. gave three distinct immunoelectrophoretic precipitin peaks against monospecific human anti-C3 serum, indicating altered C3 and in-vivo complement utilization. Altogether 45% of the 40 patients with C.F. and 15% of the normal controls had total serum levels of C3 less than 0·70 g/l (normal range of C3 0·70-1·60 g/l). Of the C.F. patients 5% had C4 serum values less than 0·13 g/l (normal range 0·13–0·50 g/l).

Discussion

Immunofluorescence staining of cryostat sections after extensive

washing to remove non-immune complexing immunoglobulins showed widespread deposits of immune complexes, especially in the respiratory and gastrointestinal tracts, but not in the kidneys from four C.F. children at necropsy and from one gut biopsy specimen of a child with C.F. It was interesting that this child also had greatly reduced serum concentrations of C3 and C4 as well as cows' milk antibodies in his serum. The strongest immunofluorescence staining was obtained with antisera to IgG, IgM, C1q, C3, and C4 though the other immunoglobulin conjugates also gave significant immunofluorescence staining of the different organs investigated. For instance, the cryostat sections of the trachea of one patient contained extensive membranous immunofluorescence to anti-IgA, IgM, IgE, C1q, C3, and C4 as well as to IgG.

Double immunofluorescence staining with two different fluorescent labels and elution with acid buffer confirmed that the immune complexes consisted of immunoglobulins as well as of complement components.

The altered form of the complement component of C3 detected in fresh C.F. plasma indicated the immunological involvement of the disease. Soothill and Hendrickse (1967) observed altered C3 component in the plasma of children with malarial nephritis who were later shown to have immune complex deposit in their kidneys and circulation. Dixon (1963) noted that antigen-excess soluble antigen-antibody complexes bound the complement component of C3 in complexed macromolecular form which was detectable in the first and second peak of SG-200 molecular sieving columns. Such macromolecular immune complexes were readily precipitated from C.F. sputum by low ionic strength solutions and were found to contain complement components as well as immunoglobulins

The reduced concentration of serum C3 in some of the C.F. patients but not of serum C4 seems analogous to the decreased concentration of C3 in about 50% of patients with chronic membranoproliferative glomerulonephritis who also invariably have either normal or raised serum C4 levels. Different values have been reported for the various complement components in C.F. serum. Nevertheless, Soothill (1967) observed that the normal levels of complement in the serum of some patients with nephritis was quite compatible with a qualitative abnormality of C3 in the patients' serum.

Autoradiographic and immunoabsorption studies showed that B.S.A. and staphylococcal antigens had made complexes with the immune complexes formed in agar gel, though the counter-immunoelectrophoretic investigations as well as the skin-hypersensitivity tests showed that several other antigens—some cross-reacting with human tissues—were also involved in the formation of immune complexes in C.F.

Analytical ultracentrifugation studies showed that the immune complexes had sedimentation coefficient between 8S and 11S. Pepys (1971) postulated that antigen-antibody complexes may be formed in the lungs on exposure to the antigen and that these complexes induce the disease process. Also, Kilburn (1973) stated that immune complexes, specifically those of anaphylaxis as well as the lipopolysaccharides of endotoxins from virus and gram-negative bacteria, produce pulmonary oedema in rabbits and man. Fluorescent anti-rabbit IgG and fluorescent anti-rat C3 stain alveolar septa and glomeruli in a linear membrane-like pattern, suggesting localization of IgG on either basement membrane or endothelial cells of alveolar capillaries and on basement membrane of glomerular capillaries in rat models of Goodpastures' syndrome (Mercola and Hagadorn, 1973).

The transient decrease of IgA and IgG coupled with low affinity and incomplete antibodies may be partly responsible for the absorptive defect of food and bacterial antigens across the gut wall and the alveolar sacs of C.F. patients. Some of the antibodies produced seemed to be cross-reacting with certain body tissues like the basement membrane of both respiratory and gastrointestinal tracts, producing a type III Arthus reaction in C.F. patients. Such tissue damage by a type III reaction in C.F. may be similar to that in extrinsic allergic interstitial alveolitis, in which the antigen absorbed from the alveolar sacs reacts with precipitating antibodies in the pulmonary capillaries, fixing complement in the process.

Ultrastructural studies of lung from C.F. patients undergoing lobectomies have shown the bronchial capillaries to have a thickened basement membrane either by duplication of the basal layer or by a deposition of a woolly or finely fibrillar material (Gulchard *et al.*, 1974). These electron-dense deposits of immune complexes in C.F. patients may be similar to those usually described in patients with nephritis with immune complex deposits.

Hypersensitivity to fungi was present in over 80% of C.F. sputum cultures, and there was also eosinophilia in 34% and high serum IgE concentrations in 23% of C.F. patients (Craceo *et al.*, 1974). In the present study the serum IgE levels were raised above 700 μg/l in 27% of the patients with C.F. Furthermore, immediate type I hypersensitivity skin reactions were found to a wide variety of antigens and common allergens including house dust mite, *Aspergillus fumigatus*, mixed moulds, cereals, mixed pollen, dog hair, egg yolk, feathers, penicillin, and dry rot. The significance of this hypersensitivity state with raised serum IgE in some patients with C.F. is not clear, but it seems similar to the condition in those patients with a congenital imbalance in their ability to secrete immunoglobulins, such that their serum contains raised IgE but not IgA, and an unusually high incidence of respiratory tract diseases. S IgA antibody molecules, which are the most abundant immunoglobulins in both respiratory and gastrointestinal secretions, cannot interact with the antigens and allergens attached to the mucosal surfaces. These circumstances may cause mast cells to react with IgE in a type I reaction that could contribute to the mortality in those C.F. patients who have developed an infection to which the patient is allergic.

Our finding of higher titres of sputum than of serum precipitins in C.F. patients was similar to observations in two patients with extrinsic allergic alveolitis caused by hypersensitivity to chickens. Thus, Warren and Tse (1974) suggested that besides offering a better diagnostic aid the sputum precipitins may be pathologically more important than the serum precipitins.

Finally, it seems that the variety of precipitins in the serum, and particularly in the sputum of C.F. patients, are related to the immune-complex deposits in the respiratory and gastrointestinal tracts—excess antigen or antibody in the circulation enhancing the formation of immune complexes. In addition, cross-reactivity of these antibodies with host tissue basement membranes seems to be a possibility in the pathogenesis of C.F. The decrease of antigen absorption from the gut by dietary control or decrease of antibody production by immunosuppressive agents could probably be important factors in the management of patients with cystic fibrosis.

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Lymphocyte Reactivity in Pregnant Women and Newborn Infants

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Summary

The mitotic response to phytohaemagglutinin (PHA) was determined in lymphocytes of mothers and their newborn infants obtained at delivery and seven days later by measuring the rate of ^{125}I-idoxuridine uptake into DNA in lymphocytes cultured in their own plasma and after washing and resuspension in fetal bovine serum. There was no difference in the unstimulated counts of maternal lymphocytes taken at delivery, whether unwashed or washed, compared with those from non-pregnant controls. With PHA stimulation the mitotic response of the maternal lymphocytes cultured in their own plasma was reduced compared with that of the control lymphocytes but washed maternal cells showed a similar response to the controls. These findings suggest that the reduced lymphocyte mitotic response to PHA in pregnancy is due to a plasma inhibitory factor. This inhibition was not evident in maternal blood taken seven days after delivery.

DNA synthesis in unstimulated cultures from newborn infants at birth and seven days after birth was greater than that in adult control cultures. With PHA stimulation the mitotic response of cord-blood lymphocytes cultured in their own plasma paralleled that of control lymphocytes but washed newborn cells showed a greater response. Thus plasma suppression similar to that observed in the mother seems also to affect infants at birth. This inhibition was not demonstrable in blood taken from infants of 7 days.

Introduction

In most pregnant women the mitotic response of their lymphocytes to phytohaemagglutinin (PHA) is reduced (Purtilo *et al.*, 1972), but the process by which this response, which seems to be predominantly the property of T cells, is reduced is uncertain (Finn *et al.*, 1972). Divergent results have been obtained regarding the suppressive effect of serum from pregnant women on the PHA response of lymphocytes from normal healthy donors (Purtilo *et al.*, 1972; Walker *et al.*, 1972). A recent study indicated the presence of a serum inhibitor in pregnancy (St Hill *et al.*, 1973) but its nature and origin is unknown. There are several conflicting reports on the response of cord-blood lymphocytes to PHA (Lindahl-Kiessling and Böök, 1964; Leikin *et al.*, 1968; Meuwissen *et al.*, 1968; Jones, 1969; Campbell *et al.*, 1975).

We have studied the mitotic responses to PHA of the lymphocytes of mothers and their newborn infants, obtained at delivery and seven days later. To investigate the presence of an inhibitory plasma factor we also compared the PHA responses of lymphocytes cultured in the presence of autologous plasma with those after washing the cells and resuspending them in fetal bovine serum.

Patients and Methods

Collection of Blood Samples.—Blood samples were obtained by venepuncture from 24 mothers at delivery, and 24 samples of cord blood were collected from their newborn infants at the same time. In each case 5 ml of blood was anticoagulated with preservative-free heparin and an EDTA sample was taken for a white cell and differential count. All samples were cultured within 24 hours of collection and in most cases within 12 hours. With each batch of samples tested control samples of venous blood, taken from age-matched healthy non-pregnant women, were treated in the same manner. Blood samples were also obtained from 13 of the mothers and their infants seven days after delivery and were similarly treated.

Preparation of Cultures.—A whole-blood microtechnique similar to that described by Maini *et al.* (1973) was used in setting up the cultures. Tubes were set up in triplicate each containing 1 ml of minimal essential medium (M.E.M.) supplemented with glutamine, non-essential amino-acids, and antibiotics to which 0·1 ml of the blood sample was added. PHA (Wellcome, dried reagent, lot K6888) was added to a concentration of 1/100. Triplicate tubes were also set up without added PHA. Preparations of washed cells were made by first diluting 2 ml of the blood sample with 20 ml of M.E.M. under which about 4 ml of heat-inactivated fetal bovine serum (Biocult, batch 000239) was layered. The cells were then centrifuged through the layer of fetal bovine serum and the autologous plasma removed by sucking off the supernatant until 2 ml remained. After resuspension of the cells 0·1 ml was added to each culture tube and similarly treated as the unwashed cell cultures.

Assessment of Lymphocyte Response to PHA.—The cultures were incubated for 72 hours at 37°C. DNA sythesis was estimated by incorporation of ^{125}I-idoxuridine (Craig *et al.*, 1969). A stock solution of this reagent (Radiochemical Centre, Amersham) was made up with added cold deoxyuridine to a concentration 10 mg/l and an activity of 20 mCi/l. From this solution 0·05 ml (1 μCi) was added to each tube three hours before the end of culture after taking off 0·5 ml of culture medium. At the end of the culture the cells were spun down and the supernatant containing the excess isotope decanted off. Red cells were lysed by washing

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